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(54) Yeast expression systems with vectors having GAPDH or PyK promoters, and synthesis of foreign proteins.

(57) Yeast cells containing DNA plasmids having foreign DNA, wherein foreign DNA is expressed, are described. For example, DNA coding for hepatitis B and its virus surface antigen (HBsAg) is ligated to a yeast plasmid to yield a product that is used to transform yeast cells. the plasmids of this invention have either GAPDH or PyK promoters, and are capable of replicating in either a yeast cell or a bacterial cell.

EP 0 120 551 A2

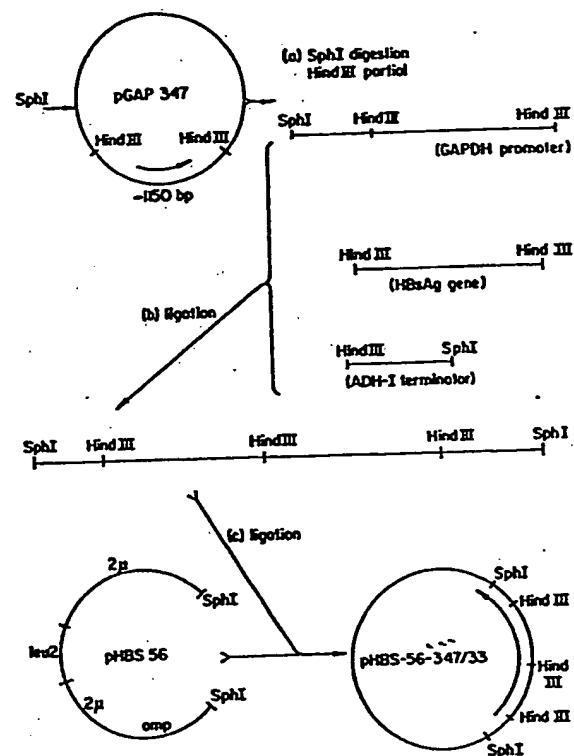


FIG. 3.

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YEAST EXPRESSION SYSTEMS WITH VECTORS HAVING GAPDH
OR PyK PROMOTERS, AND SYNTHESIS OF FOREIGN PROTEIN

5

BACKGROUND OF THE INVENTION

For maximal expression of foreign genes in microbial systems it is usually advantageous to employ homologous regulatory elements within the expression vector. Efficiency of expression (product formation) is believed to be a function of and proportional to the strength of the promoter employed. In addition, regulation of gene expression by nutritional factors under the control of the experimenter offers a further useful manipulatory tool. The glycolytic enzyme genes of yeast, e.g., those coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PyK), possess the above useful properties, i.e., high levels of expression (and thus by inference very efficient promoters) and susceptibility to regulation by components of the growth medium. For example, GAPDH can comprise as much as 5% of the dry weight of commercial baker's yeast (Krebs, E.G., J. Biol. Chem. (1953) 200:471). Furthermore, these enzymes are also highly inducible. For example, when yeast cultures are shifted from growth on acetate to glucose, the activity of GAPDH increased up to 200-fold in proportion to the concentration of the sugar in the medium (Maitra, P.K. and Lobo, Z., J. Biol. Chem. (1971) 246:475). These results suggest that the transcriptional machinery of these genes is highly regulated, perhaps by the participation of DNA sequences present in the 5' non-coding flanking region of the genes.

This invention relates to the isolation, structure and the successful use in yeast expression plasmids of DNA fragments corresponding to the 5' non-coding regions of the regulatable yeast genes GAPDH and PyK. These fragments which contain DNA sequences

with strong transcription-promoting activity are called "promoters". They are ideal components of DNA vectors for commercial production of large quantities of protein coded by foreign genes under their transcriptional control.

In addition, this invention encompasses yeast expression plasmids further comprising an appropriate terminator to form a "cassette" of promoter-foreign gene-terminator. The presence of the terminator increases expression of the foreign DNA.

An early attempt to express foreign DNA in yeast failed (Beggs, J.D. et al., Nature (1980) 283:285). In this report, the hemoglobin DNA (inserted with its own promoter) was transcribed but the RNA was not spliced. A variety of explanations for this result are possible, e.g., an incorrect location for the initiation of transcription and/or the poor ability of yeast cells to carry out splicing of intervening sequences (introns).

Three GAPDH genes of yeast have been cloned (Holland, M.J. et al., Basic Life Science (1981) 19:291), but their promoters have not been used for constructing expression systems in yeast by recombinant DNA methods. The PyK gene has also been cloned, but by genetic complementation only (no structural studies performed) (Kawasaki, G. and Fraenel, D.G., Biochem. Biophys. Res. Comm. (1982) 108:1107). Other yeast promoters, e.g., that of alcohol dehydrogenase I (Valenzuela, P. et al., Nature (1982) 298:347 and Hitzeman, R.A. et al., Nature (1981) 293:717) and phosphoglycerate kinase (Tuite, M.F. et al., EMBO J. (1982) 1:603 and Hitzeman, R.A. et al., Science (1983) 219:620) have been linked to foreign genes to produce yeast expression but no terminators were used. The present invention provides new promoters for yeast expression systems and combines the advantages of

highly expressive promoters with the enhanced expression found with appropriately ligated terminators.

BRIEF DESCRIPTION OF THE INVENTION

This invention relates to a yeast expression
5 vector comprising a segment of foreign DNA, e.g., that
coding for hepatitis B virus (HBV) surface antigen
(HBsAg), under transcriptional control of either a
yeast GAPDH promoter or a yeast PyK promoter. Termina-
tors may also be appropriately attached. The expression
10 vector typically has a yeast replication origin and a
bacterial replication origin and is capable of repli-
cating in either type of cell. The expression vector,
when used to transform yeast cells, will yield
substantial amounts of the protein coded by the segment
15 of foreign DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Isolation and tailoring of a GAPDH
promoter fragment.

Fig. 2: DNA sequence of the GAPDH promoter
20 fragment.

Fig. 3: Construction of a yeast expression
plasmid containing the GAPDH promoter.

Fig. 4: Nucleotide sequence of the pyruvate
kinase (PyK) gene.

25 Fig. 5: Construction of a yeast expression
plasmid containing the PyK promoter region.

DETAILED DESCRIPTION OF THE INVENTION

In principle, yeast expression plasmids have
particular advantages, including the following. Yeast
30 can be grown in large-scale culture for commercial
production by processes well-known in the art. In
contrast, bacteria in large-scale culture are subject
to the frequent problem of "phage-out". Yeast also
appears to have much the same ability as mammalian
35 cells to add carbohydrate groups to newly synthesized
proteins, a capacity that bacteria do not have. Now

that cDNA sequences are readily obtainable, the problem of expressing genes having introns is easily avoided.

The vectors of the present invention encompass promoters of unusually high efficiency. A promoter is defined herein as a DNA segment capable of functioning to initiate transcription of an adjoining DNA segment. Transcription is the synthesis of RNA (herein termed messenger RNA or mRNA), complementary to one strand of the DNA adjoining the promoter region. In eukaryotes, messenger RNA synthesis is catalyzed by an enzyme termed RNA polymerase II. The minimum essential elements of promoter function are the following: To provide a starting point for the initiation of transcription and to provide a binding site for RNA polymerase II near the start site permitting selection of the proper strand of DNA as a template for messenger RNA synthesis. In addition, a eukaryotic promoter functions to regulate the relative efficiency of transcription of coding segments under its control. An active promoter is one which elicits synthesis of relatively large amounts of mRNA complementary to a strand of the adjacent DNA coding segment.

The structural correlates of promoter function have not been clearly established. A promoter segment usually can be identified in nature as a region lying adjacent to the 5'-end of a given structural gene. (References to the 5' and 3' ends of a gene will be understood to indicate the corresponding respective ends of mRNA transcribed therefrom, and these, in turn, will be understood to correlate with the NH₂- and -COOH termini of the encoded protein, respectively.) Comparisons of the nucleotide sequences of promoters for various genes from various species have revealed only a few short regions of nucleotide sequence similarity in common among them. Most notable of these is the "TATA Box," a segment of about 5 to 10 nucleotides located generally about 70 to 230 nucleotides upstream

from the site of transcription initiation, having a sequence generally resembling TATAA. For review of structural comparisons see Breathnach, R. and Chambon, P., Ann. Rev. of Biochem. (1981) 50:349. The TATA Box
5 is believed to function in initiation of transcription.

The foreign gene will be free or substantially free of codons from the normal structural gene associated with the promoter. Usually, the foreign gene will be joined to a non-coding 3'-end of the regulatory
10 region encompassing the promoter, so as to be free of the amino acids at the N-terminus of endogenous gene naturally associated with the regulatory region. That is, fewer than about 3 codons (9 nucleotides) will be retained with the regulatory region when joined to the
15 foreign gene.

The presence of the terminator sequence at the 3' end of the coding segment enhances expression. The effect is generally similar to the addition of rho factor to prokaryotic transcription systems, wherein
20 the rate of the release of RNA polymerase is enhanced to produce an increase in the rate of reinitiation of transcription. It will be understood that, while the terminator sequences are not required for detectable expression of foreign DNA segments, it is preferable to
25 appropriately link them to enhance expression. The terminator region may be naturally associated with the same or different structural gene as the promoter region.

The most appropriate DNA vector for the GAPDH or PyK construction of this invention is a shuttle
30 vector. These vectors can "shuttle" between a bacterial strain, such as E. coli, and yeast, since they have a bacterial origin of replication and a yeast origin of replication, see, e.g., Ammerer, G. et al., Recombinant DNA, Proc. Third Cleveland Symposium Macromolecules
35 (Walton, A.G., ed.), p. 185, Elsevier, Amsterdam (1981). A typical bacterial origin of replication is

derived from, e.g., pBR322. The most useful yeast origin of replication is found in the extrachromosomal genetic element known as the 2 micron circle. In laboratory strains the 2 micron plasmid DNA is found in approximately 50 copies per cell and is stably maintained. For a review, see, for example, Curr. Topics Micro. Imm. (1982) 96:119. This yeast plasmid has also been sequenced (Hartley, J.L. et al., Nature (1980) 286:860).

Representative samples of the plasmids and host cells used in the constructions of this invention have been placed on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. Plasmid pPyK 9.1.1 and yeast cell transformants 2150-2-3/pHBS-56 GAP347/33 and 2150-2-3/pHBS56PyK were placed on deposit on February 18, 1983 and have received ATCC Accession numbers 40061, 20665 and 20666, respectively.

In the Examples that follow, many of the techniques, reactions and separation procedures are already well-known in the art. All enzymes, unless otherwise stated, are available from one or more commercial sources, such as New England Biolabs, Beverly, Massachusetts; Collaborative Research, Waltham, Massachusetts; Miles Laboratories, Elkhart, Indiana; Boehringer Biochemicals, Inc., Indianapolis, Indiana and Bethesda Research Laboratories, Rockville, Maryland. Buffers and reaction conditions for restriction enzyme digestion were used according to recommendations supplied by the manufacturer for each enzyme, unless otherwise indicated. Standard methodology for other enzyme reactions, gel electrophoresis separations and E. coli transformation may be found in Methods in Enzymology, (1979) 68. Transformation of yeast protoplasts can be carried out essentially as described by Beggs, Nature (1978) 275:104.

E. coli strains useful for transformation include X1776; K12 strain 294 (ATCC No. 31446); RR1 and HB101. Yeast strains XV610-8c having the genotype (a ade2 ade6 leu2 lys1 trp1 can1) and GM-3C-2, genotype: 5 (Leu2 Trp1 His4 CYC1-1CYP3-1) (Faye, G. et al., Proc. Natl. Acad. Sci. (1981) 78:2258) can be typically used for yeast transformations. It would be understood, however, that virtually any strain of yeast is useful for transformation. Bacteria can be grown and selected 10 according to procedures described by Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972). Yeast can be grown on the following media: YEPD containing 1% (w/v) yeast extract, 2% (w/v) peptone and (w/v) 15 glucose; and, in the case of plating medium, 3% (w/v) agar. YNB plus CAA contains 6.7 grams of yeast nitrogen base (Difco Laboratories, Minneapolis, Minnesota), 10mg of adenine, 10mg of uracil, 5g casamino acids (CAA) (Difco), 20g glucose; and, in the case of plating 20 media, 30g agar per liter. Selection for tryptophan prototrophy can be made on plates containing 6.7g yeast nitrogen base (lacking amino acids), supplemented for all growth requirements of the strain to be transformed except tryptophan.

25

EXAMPLE 1

Cloning of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

A complementary DNA (cDNA) containing the 30 yeast GAPDH coding sequences was prepared in the following manner:

PolyA+ RNA was isolated from yeast strain A364A. Double-stranded cDNA was synthesized using AMV reverse transcriptase and E. coli DNA polymerase I. 35 Poly-dC-tails were added to the double-stranded cDNA molecule using deoxynucleotide terminal transferase. Poly-dC-tailed cDNA was annealed to poly-dG-tailed

pBR322 and used to transform E. coli HB101. One thousand transformants were screened by colony hybridization to labeled PolyA+ RNA, and a subset further examined by restriction endonuclease mapping, and DNA sequencing. Three clones containing GAPDH sequences were isolated from the pool. One clone (pcGAP-9) contained an insert of about 1200 base pairs (bp) and was used for further work.

A yeast gene library was prepared by inserting fragments obtained after partial digestion of total yeast DNA with restriction endonuclease Sau3A into lambda phage Charon 28, according to Blattner, F.R. et al., Science (1977) 196:161-169. Several fragments containing yeast GAPDH coding sequences were isolated by screening the phage library with labeled DNA from pcGAP-9. The yeast GAPDH gene of one of these clones was subcloned in pBR322 as a 2.1kb HindIII fragment (pGAP-1, see Fig. 1) or as a 3.5kb BamHI fragment (pGAP-2). The GAPDH promoting-active fragments were isolated from these clones. The HindIII-HhaI fragment of about 800bp was ligated to the HhaI-HindIII fragment of about 350bp. The resulting 1061bp HindIII fragment was isolated by gel electrophoresis and cloned in pBR322, (pGAP-347), and the sequence determined (see Fig. 2).

EXAMPLE 2

Construction of yeast vectors containing the GAPDH promoter, active in the expression of HBsAg.

A plasmid vector (pHBS-56GAP347/33), for the expression of HBV surface antigen in yeast, using the GAPDH promoter fragment was constructed as depicted in Fig. 3.

Total digestion of pGAP-347 with SphI followed by partial digestion with HindIII yielded an approximately 1700bp SphI-HindIII fragment having about 1060bp of GAPDH promoter and about 530bp of pBR322. The

1700bp SphI-HindIII GAPDH promoter fragment was ligated with the 840bp HindIII-HindIII fragment (containing the HBsAg coding region, 26 bases of 5' non-coding region and 128bp of 3' non-coding region, obtained from
5 pHBS-56) and then with the 350bp HindIII-SphI fragment containing the ADH-1 termination region (isolated from pHBS-56). The 2900bp SphI fragment (cassette) was isolated and cloned in pHBS-56 previously digested with SphI. The plasmid pHBS-56 (ATCC Accession No. 40047)
10 has been described in a co-pending application (EPA No. 82.401473.2 published as no. 72318, of Regents of the University of California, herein incorporated by reference) and contains the entire 2 micron plasmid, in addition to a region with the yeast leu2 gene and the
15 amp resistance locus of pBR322. The resulting plasmid (pHBS-56GAP347/33) in which the promoter, gene and termination regions were in the proper orientations was isolated and used to transform yeast strain AB102 (MATa, pep 4-3, leu 2-3 leu2-112, ura 3-52, his 4-580, cir^o) or strain 2150-2-3 (MATa, adel, leu2-04, cir^o).
20 Strain AB102 is derived from SF657-9c by curing of 2 micron plasmids. Strain 2150-2-3 is from the collection of Dr. Leland Hartwell at the University of Washington.

25

EXAMPLE 3Synthesis of HBsAg in yeast under GAPDH promoter control (plasmid pHBS-56GAP347/33).

One hundred ml cultures of strain AB102 containing plasmid pHBS56-347/33 were grown to optical
30 density at 650nm of 1. Cell-free lysates were prepared by agitation with glass beads and removal of cell debris by centrifugation. HBsAg was measured by the Abbott AusriaII radioimmunoassay and protein concentration was determined by the Coomassie blue binding
35 method. The results are shown in Table 1. They indicate that the GAPDH promoter is about 5 times more

effective than the ADH-1 promoter for protein product expression in yeast.

Table 1: Synthesis of HBsAg in yeast

(a) control from pHBS-56 (ADH-I promoter)				
5	<u>Exp#</u>	<u>sAg</u> (μ g/ml)	<u>protein</u> (mg/ml)	<u>Spec. Activity</u> (μ gsAg/mg protein)
	1	8.8	18	0.49
	2	14	25	0.56
	3	12.4	20	0.62
10	(b) from pHBS-56GAP347/33 (GAPDH promoter)			
	<u>Exp#</u>	<u>sAg</u> (μ g/ml)	<u>protein</u> (mg/ml)	<u>Spec. Activity</u> (μ gsAg/mg protein)
	1	36	14	2.6
	2	35	12	2.9
15	3	37	12.5	3.0

Similar results were obtained by substituting yeast strain 2150-2-3 for yeast strain AB102 and repeating Example 3.

EXAMPLE 4

20 Cloning of the yeast pyruvate kinase gene.

The pyruvate kinase gene was cloned by complementation. A yeast pyruvate kinase minus mutant was transformed with a pool of recombinant YEp24 plasmids containing wild type yeast genomic DNA. The yeast strains S288C (genotype: SUC2, mal, gal2, CUP1) and pyk 1-5 (genotype: a, adel, leu1, met14, ura3, pyk1-5) were obtained from the Yeast Genetic Stock Center, Department of Biophysics, University of California, Berkeley. The yeast genomic bank used consists of a partial Sau3A digest of total DNA from

the strain S288C cloned into the BamHI site of the "shuttle" vector YEp24. The vector YEp24 contains pBR322 sequences for selection and growth in bacteria, the yeast URA3 gene for selection in yeast and an EcoRI fragment of the yeast 2 μ circle to ensure plasmid replication and segregation in yeast. The pool includes sufficient independent recombinant plasmids to represent the entire yeast genome.

The strain *pyk1-5* is unable to grow on medium containing glucose or lacking uracil because of mutations in this strain at the *Pyk1* and *URA3* loci, respectively. Transformation of this strain with the YEp24 genomic library and selection for transformants which are able to grow on medium lacking uracil and containing glucose selects for those cells which have acquired YEp24 containing the pyruvate kinase gene. Transformation of 3.5×10^8 *pyk1-5* yeast cells with 10 μ g of YEp24 recombinant plasmid pool DNA yielded 5 independent transformants which grew in the absence of uracil and the presence of glucose.

Characterization of the insert DNA of these transformants by restriction enzyme analysis indicated that they contained overlapping DNA inserts. We focused on a single transformant, pPyK 9.1, which contained a 7.0kb insert. The pyruvate kinase gene was localized within this insert by determining which insert-specific restriction fragments hybridized to a mRNA of about 1.7kb expected for the pyruvate kinase mRNA. The localization of the *PyK* gene was confirmed by subcloning appropriate regions of the insert DNA and observing complementation of function in the *pyk1-5* mutant. A subclone pPyK 9.1.1 which contained the *PyK* gene on a 4.4kb insert was sequenced and used in expression plasmid constructions.

EXAMPLE 5Sequence of the yeast pyruvate kinase gene.

A total of 2885 nucleotides of the PyK gene have been sequenced including 1497 nucleotides in a single open reading frame, 911 nucleotides of 5' untranslated region and 477 nucleotides of 3' untranslated region (see Fig. 4). The gene encodes a polypeptide of 499 amino acids to give a monomer molecular weight of 54,608 daltons which agrees well with the expected value for yeast PyK. The amino acid composition derived from the nucleotide sequence also corresponds closely with that measured from the isolated yeast protein. The nucleotide sequence predicts a carboxy terminal valine which has been found for yeast pyruvate kinase.

EXAMPLE 6Construction of yeast expression plasmids using the pyruvate kinase promoter region.

Two different constructions were made: pHBS16 PyK and pHBS56 PyK. The procedures are outlined in Fig. 5.

The plasmid pPyK 9.1.1, which contains the yeast PyK gene cloned in pBR322 was digested with XbaI and the protruding ends filled in with deoxynucleotides using DNA polymerase I. The product was digested with BamHI to finally isolate a 912bp BamHI-blunt fragment containing the PyK promoter and 8 bases from the PyK coding region. This fragment was ligated to plasmid pHBS-6 (contains the HBSAg gene, in which the 5' non-coding region has been deleted, cloned in pBR322) previously digested with NcoI, filled in using DNA polymerase and digested with BamHI. After transformation of E. coli, pHBS-6PyK was isolated. This plasmid contains the PyK promoter with codons for 3 extra amino acids fused in phase with the HBSAg coding sequence,

ATGTCTAG, CATG, .

pHBS-6PyK was digested with BamHI to completion and partially digested with EcoRI to isolate a 1750bp BamHI-EcoRI fragment containing the PyK promoter fused to the HBsAg gene. This 1750bp fragment was ligated to
5 the large fragment obtained after digestion of pHBS-16 (ATCC Accession No. 40043, plasmid described in European Patent Application No. 82.401473.2 mentioned above)

with BamHI

10 and EcoRI and used to transform E. coli. The yeast expression plasmid pHBS-16PyK was obtained. pHBS-16PyK was digested to completion with SphI and XbaI and a 1200bp SphI-XbaI fragment (containing 200bp of pBR322, the PyK promoter and 100bp of the 5' region of the
15 HBsAg gene) was isolated. This 1200bp SphI-XbaI fragment was ligated to a 1070bp XbaI-SphI fragment (isolated from pHBS-56) containing the 3' end of the HBsAg gene and the ADH-1 terminator. After digestion with SphI, a SphI-SphI 2300bp fragment (cassette)
20 containing the PyK promoter, HBsAg gene and ADH-1 terminator was isolated. This cassette fragment was cloned in pHBS-56 which had been previously digested with SphI. The yeast expression plasmid pHBS-56 PyK was obtained. This plasmid was used to transform yeast
25 strain AB102 (see Example 2) or strain 2150-2-3 (see Example 2).

EXAMPLE 7

Synthesis of HBsAg in yeast under PyK promoter control.

30 One hundred ml cultures of strain AB102 containing plasmid pHBS-56 PyK were grown to optical densities at 650nm of 1-2. Cell-free lysates were prepared by agitation with glass beads and removal of cell debris by centrifugation. HBsAg was measured by
35 the Abbott AusriaII radioimmunoassay and protein concentration was determined by the Coomassie blue binding method. The results are shown in Table 2.

They indicate that PyK promoter is at least two times more efficient than the ADH1 promoter for expression of protein product in yeast.

Table 2: Synthesis of HBsAg in yeast

5 (a) from pHBS-56 (control, ADH-I promoter)

<u>Exp#</u>	<u>sAg</u> (μ g/ml)	<u>protein</u> (mg/ml)	<u>Spec. Activity</u> (μ gSAg/mg protein)
1	8.2	24	0.34
2	7.2	24	0.32
10 3	4.7	27	0.23

(b) from pHBS-56 PyK (PyK promoter)

<u>Exp#</u>	<u>sAg</u> (μ g/ml)	<u>protein</u> (mg/ml)	<u>Spec. Activity</u> (μ gSAg/mg protein)
1	18	2.5	0.68
15 2	10.6	22	0.48
3	15.2	27	0.56

Similar results were obtained by substituting yeast strain 2150-2-3 for yeast strain AB102 and repeating Example 7.

CLAIMS

1. A yeast expression vector comprising a segment of foreign DNA under transcriptional control of a yeast glyceraldehyde-3-phosphate dehydrogenase promoter, said segment being in the correct orientation for transcription and substantially free of codons from yeast glyceraldehyde-3-phosphate dehydrogenase at the 5'-end of said foreign DNA.
2. A yeast expression vector comprising a segment of foreign DNA under transcriptional control of a yeast pyruvate kinase promoter, said segment being in the correct orientation for transcription and substantially free of codons from yeast pyruvate kinase at the 5'-end of said foreign DNA.
3. A yeast expression vector of claim 1 or claim 2 further comprising a terminator attached to the 3' end of the segment of foreign DNA.
4. A yeast expression vector according to any of claims 1 and 2, further comprising yeast two micron plasmid DNA or portion thereof.
5. A yeast expression vector of any claims 1 and 2 wherein said foreign DNA codes for hepatitis B surface antigen or portion thereof.
6. The plasmid pHBS-56GAP347/33.
7. The plasmid pHBS-56 PyK.
8. A method of expressing a DNA coding segment in yeast, comprising the steps of:
 - (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast glyceraldehyde-3-phosphate dehydrogenase promoter substantially free of codons from the 5'-end of yeast glyceraldehyde-3-phosphate dehydrogenase, said promoter being adjacent to the 5' end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and

- (b) transforming yeast cells with the coding segment expression vector.
9. A method of expressing a DNA coding segment in yeast, comprising the steps of:
- 5 (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast pyruvate kinase promoter substantially free of codons from the 5'-end of the yeast pyruvate kinase, said promoter being adjacent
- 10 to the 5'-end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and
- (b) transforming yeast cells with the coding
- 15 segment expression vector.
10. A method according to any one of claims 8 and 9 wherein said yeast expression vector further comprises a terminator attached to the 3' end of the inserted DNA coding segment.
- 20 11. A method according to any of claims 8 and 9 wherein said yeast expression vector further comprises a bacterial cell replication origin and is capable of replicating in a bacterial cell.
12. A method according to claim 10 wherein said
- 25 terminator comprises the yeast alcohol dehydrogenase I terminator.
13. A method according to claim 10 wherein said terminator comprise the yeast GAPDH terminator.
14. A method according to claim 10 wherein said
- 30 terminator comprises the yeast PyK terminator.

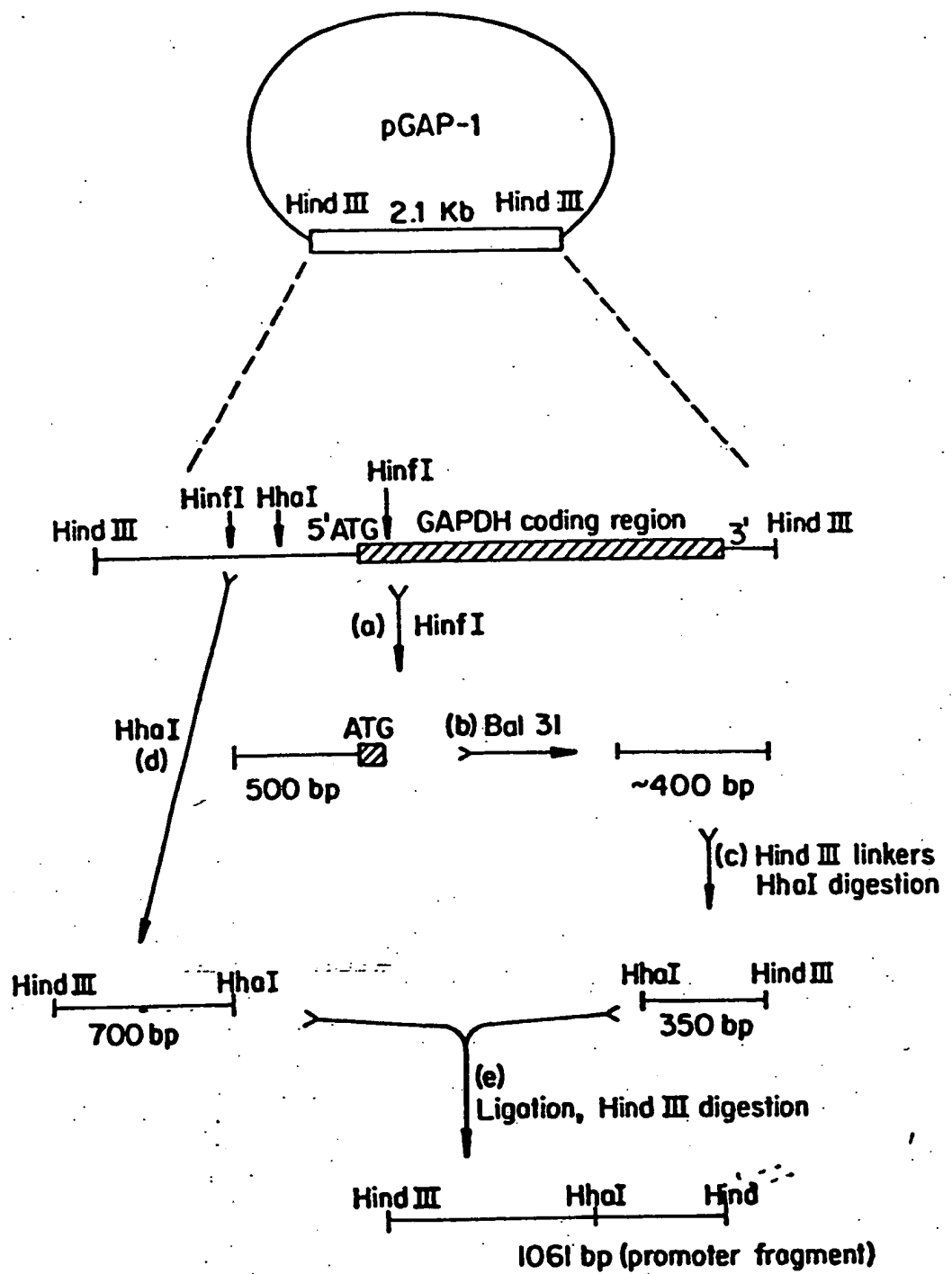


FIG. 1.

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DNA 347

10 20 30 40 50 60
 AAGCTTACCA GTTCTCACAC GGAACACCAC TAATGGACAC AAATTCGAAA TACTTTGACC
 70 80 90 100 110 120
 CTATTTTCGA GGACCTTGTC ACCTTGAGCC CAAGAGAGCC AAGATTITAA TTTTCTATG
 130 140 150 160 170 180
 ACTTGAIGCA AATTCCCAAA GCTAATAACA TGCAAGACAC GTACGSTCAA GAAGACATAT
 190 200 210 220 230 240
 TTGACCTCTT AACTGGTTCA GACGCGACTG CCTCATCAGT AAGACCCGTT GAAAAGAAGCT
 250 260 270 280 290 300
 TACCTGAAAA AAACGAATAT ATACTAGCGT TGAATGTTAG CGTCAACAAC AAGAAGTTTA
 310 320 330 340 350 360
 ATGACGCGGA GGCCAAGGCA AAAAGATTCC TTGATTACGT AAGGGAGTTA GAATCATTTT
 370 380 390 400 410 420
 GAATAAAAAA CACGCTTTTT CAGTTCGAGT TTATCATTAT CAATACTGCC ATTTCAAAGA
 430 440 450 460 470 480
 ATACGTAAAT AATTAATAGT AGTGATTTTC CTAACCTTAT TTAGTCAAAA ATTAGCCTTT
 490 500 510 520 530 540
 TAATTCGCT GTAACCCGTA CATGCCCAAA ATAGGGGGCG GGTTACACAG AATATATAAC
 550 560 570 580 590 600
 ATCGTAGGTG TCTGGGTGAA CAGTTTATCC CTGGCATCCA CTAATATATA TGGAGCTCGC
 610 620 630 640 650 660
 TTTAAGCTG GCATCCAGAA AAAAAAGAA TCCAGCACC AAAATATTGT TTTCTTCACC
 670 680 690 700 710 720
 AACCATCAGT TCATAGGTCC ATTCTCTTAG CGCAACTACA GAGAACAGGG GCACAAACAG
 730 740 750 760 770 780
 GCAAAAAACG GGCACAACCT CAATGGAGTG ATGCAACCTG CCTGGAGTAA ATGATGACAC
 790 800 810 820 830 840
 AAGGCAATTG ACCCAGCAT GTATCTATCT CATTTTCTTA CACCTTCTAT TACCTTCTGC
 850 860 870 880 890 900
 TCTCTCTGAT TTGAAAAAG CTGAAAAAA AGGTTGAAAC CAGTTCCCTG AAATTATTCC
 910 920 930 940 950 960
 CCTACTTGAC TAATAAGTAT ATAAAGACGG TAGGTATTGA TTGTAATTCT GTAAATCTAT
 970 980 990 1000 1010 1020
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FIG. 2

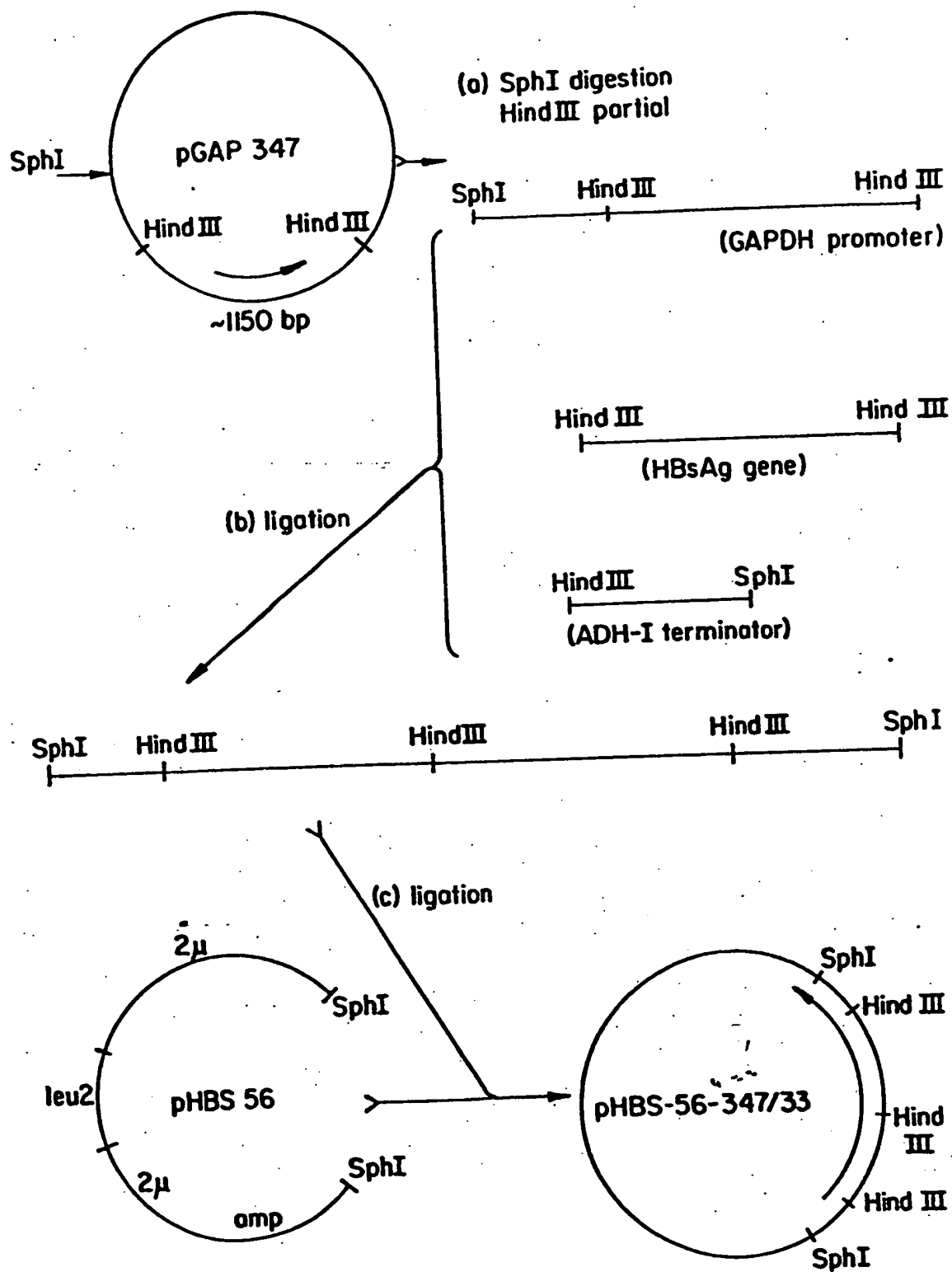
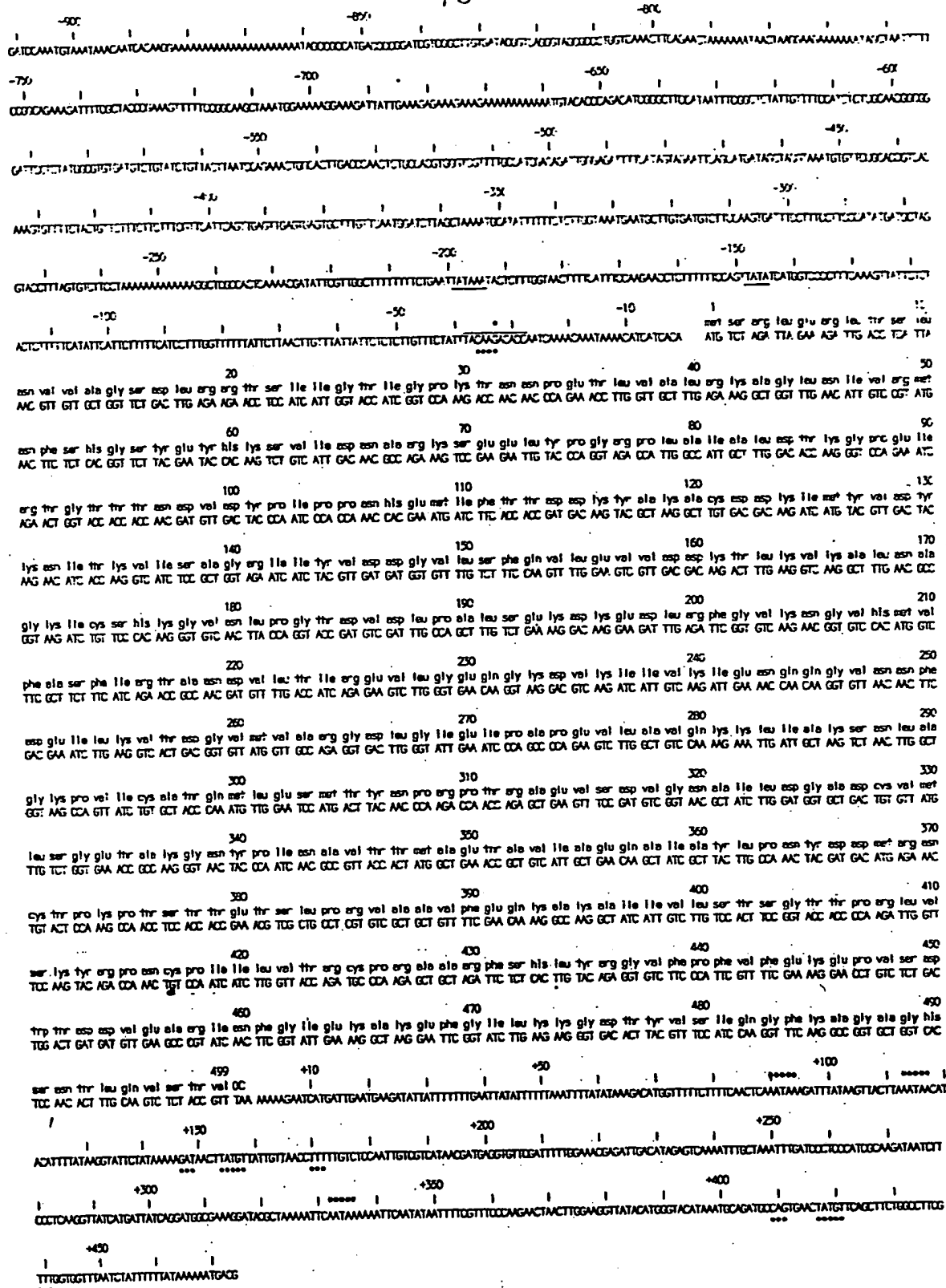


FIG 3



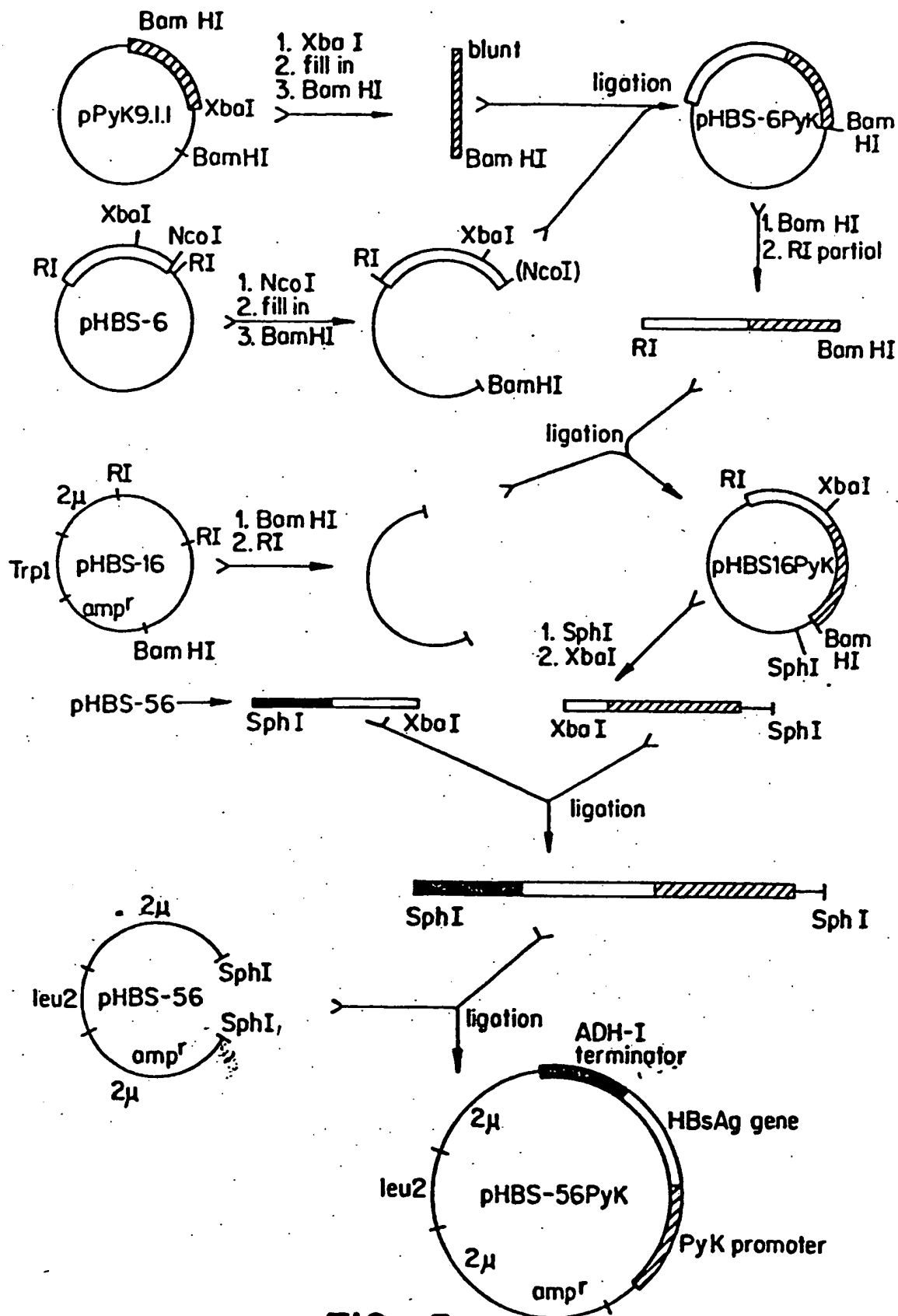


FIG. 5.

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- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
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- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
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